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Characterization of steady-state activities of cytochrome *c* oxidase at alkaline pH: mimicking the effect of K-channel mutations in the bovine enzyme

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Abstract

The cytochrome *c* oxidase activity of the bovine heart enzyme decreases substantially at alkaline pH, from 650 s^{-1} at pH 7.0 to less than 10 s^{-1} at pH 9.75. In contrast, the cytochrome *c* peroxidase activity of the enzyme shows little or no pH dependence (30–50 s^{-1}) at pH values greater than 8.5. Under the conditions employed, it is demonstrated that the dramatic decrease in oxidase activity at pH 9.75 is fully reversible and not due to a major alkaline-induced conformational change in the enzyme. Furthermore, the K_m values for cytochrome *c* interaction with the enzyme were also not significantly different at pH 7.8 and pH 9.75, suggesting that the pH dependence of the activity is not due to an altered interaction with cytochrome *c* at alkaline pH. However, at alkaline pH, the steady-state reduction level of the hemes increased, consistent with a slower rate of electron transfer from heme *a* to heme a_3 at alkaline pH. Since it is well established that the rate of electron transfer from heme *a* to heme a_3 is proton-coupled, it is reasonable to postulate that at alkaline pH, proton uptake becomes rate-limiting. The fact that this is not observed when hydrogen peroxide is used as a substrate in place of O_2 suggests that the rate-limiting step is proton uptake via the K-channel associated with the reduction of the heme a_3/Cu_B center prior to the reaction with O_2. This step is not required for the reaction with H_2O_2, as shown previously in the examination of mutants of bacterial oxidases in which the K-channel was blocked. It is concluded that at pH values near 10, the delivery of protons via the K-channel becomes the rate-limiting step in the catalytic cycle with O_2, so that the behavior of the bovine enzyme resembles that of the K-channel mutants in the bacterial enzymes.

Keywords: Cytochrome *c* oxidase; Cytochrome *c*; Peroxidase; Steady-state kinetics; Circular dichroism; Alkaline pH value

1. Introduction

Cytochrome *c* oxidase (COX)(EC 1.9.3.1) is the terminal enzyme in the respiratory chains of mitochondria and aerobic bacteria, which oxidizes ferrocytochrome *c* and reduces molecular oxygen to water [1,2]. The enzyme conserves the free energy of the reaction by pumping protons vectorially across the membrane from the mitochondrial matrix to the intermembrane space [3]. For each electron transported from ferrocytochrome *c* to dioxygen (four in total), two protons are taken up by COX from the mitochondrial matrix. One proton is used in the reduction of

Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CD, circular dichroism; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; COX, cytochrome *c* oxidase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); (H)EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); LM, dodecyl β-D-maltoside (lauryl maltoside); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride; UV, ultra-violet

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dioxygen to water, and the other is vectorially translocated across the inner mitochondrial membrane.

Bovine heart COX consists of 13 polypeptide subunits in a 1:1 stoichiometry [4]. COX contains four redox-active metal centers, three of which (heme *a*, heme a_3, and Cu_B) are located in subunit I, with the remaining redox center, the dinuclear Cu_A, residing in the solvent exposed portion of subunit II [5]. The active site is the heme a_3/Cu_B binuclear center which is the binding site of the substrates, oxygen or hydrogen peroxide [6,7]. The primary binding site for ferrocytochrome *c* is located on subunit II and electrostatic interactions between amino groups of lysine from the substrate and carboxyl groups from subunit II are thought to align the substrate for maximum electron transfer activity [8,9].

The routes of proton uptake through the enzyme are clearly defined [5]. Two channels, the "K-channel" [10] and the "D-channel" [11–13], are involved in proton uptake from the mitochondrial matrix during specific steps of the COX catalytic cycle. The K-channel appears to be required only for electron transfer steps prior to the interaction with dioxygen, in which the fully oxidized enzyme is reduced by two reducing equivalents. The D-channel is required for electron transfer steps after formation of an oxygenated enzyme intermediate (for a review, see Ref. [14]). Two oxygenated intermediates have been characterized, species P and F. The sequence observed is $O \rightarrow R_2 \rightarrow P \rightarrow F \rightarrow O$, in which the oxidized enzyme (O) is reduced by two electrons (R_2), reacts with O_2 to yield the oxygenated intermediate P, which is converted by two one-electron transfer reactions to the F state and, finally, back to the O state. This is shown schematically in Fig. 1.

When the enzyme uses hydrogen peroxide (rather than dioxygen) as a substrate, the initial "pre-reduction" to form the R_2 state is bypassed, since the H_2O_2 itself is two electrons reduced compared to O_2 [15]. As shown in Fig. 1, this "peroxide shunt" eliminates the steps in the reaction which utilize the K-channel. This was demonstrated by analysis of site-directed mutants that block the K-channel in the *Rhodobacter sphaeroides* oxidase [16–18]. In contrast, mutations that block the "D-channel" completely eliminate peroxidase activity as well as the reaction with O_2 [19].

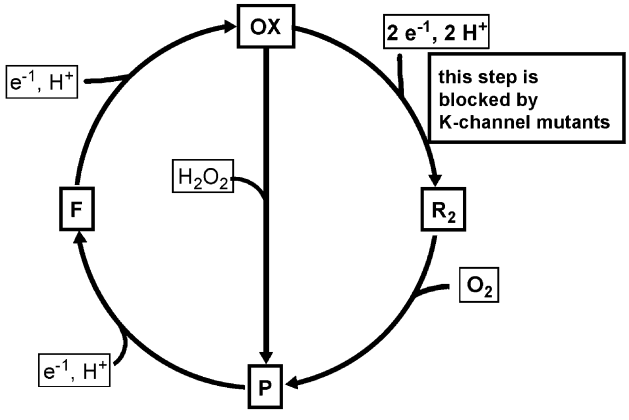

Fig. 1. Schematic diagram of the catalytic cycles of cytochrome *c* oxidase. The catalytic cycles of the enzyme for oxygen and hydrogen peroxide as substrates are presented. Intermediate E (formed by a one-electron reduction), which occurs between intermediates O_x (O), was omitted for simplicity.

It has been shown previously that the oxidase activity of the bovine enzyme exhibits a steep decrease above pH 8.0, and it was proposed [20,21] that this is due to an increase in the K_m of ferrocytochrome *c*. In the current work, it is demonstrated that the decrease in steady-state activity is not due to a change in the interaction between the oxidase and ferrocytochrome *c*, but is due to a slow rate of electron transfer from heme *a* to heme a_3. Since no decrease in the peroxidase activity of the enzyme is observed in the same range of pH values, the most likely explanation is that at very alkaline pH, proton delivery through the K-channel becomes rate-limiting for the reaction with O_2. This step is not required for the reaction with H_2O_2, so there is no significant pH-dependent drop in the peroxide activity.

2. Materials and methods

2.1. Enzyme preparations

Mitochondria were isolated from bovine heart using the method of Azzone et al. [22] and the oxidase was prepared as described previously [23]. This preparation has been previously demonstrated to be in a monomeric form [24]. Monomeric enzyme has been shown to have distinct kinetic features in single turnover experiments [25], but has unaltered steady-state electron transfer and proton pumping activities [26]. Purity was assessed as described previously [26], protein concentration was determined by the method of Lowry et al. [27], and heme aa_3 concentration was determined using a reduced minus oxidized extinction coefficient of 24 mM^{-1} cm^{-1} at 605 nm [28].

Horse heart ferrocytochrome *c* (Sigma, type VI) was prepared as described previously [29]. Reduction and elution were performed at the intended pH and ionic strength of the subsequent assays. An extinction coefficient of 29.5 mM^{-1} cm^{-1} at 550 nm was used for ferrocytochrome *c* concentration [30]. The alkaline form of ferricytochrome *c* at pH 9.75 was monitored at 695 nm [31].

Phospholipid vesicles containing the oxidase were prepared by sonication in 0.1 M CHES NaOH, pH 9.5, as described by Nguyen et al. [32]. Respiratory control ratios of these preparations measured spectrophotometrically at pH 9.75 were routinely 3–5 and the orientation of the cytochrome *c* binding domain facing the outside was approximately 85% [32].

2.2. Steady-state kinetics of ferrocytochrome c oxidation by bovine oxidase

COX was incubated for 1 h on ice in lauryl maltoside (LM) [5 mg LM/mg protein] and the dependence of the rate

of oxidation of ferrocytochrome *c* on the concentration of cytochrome *c* was measured under four conditions of pH and ionic strength. At pH 7.8 and an ionic strength of 10 mM, both assay and solubilization buffers consisted of 100 mM sucrose, 1.7 mM KCl, 1 mM LM, and 10 mM HEPES NaOH, pH 7.8, while at pH 7.8 and 250 mM ionic strength, these buffers were 100 mM sucrose, 212.5 mM KCl, 1 mM LM, and 45 mM HEPES NaOH, pH 7.8. At pH 9.75 and 10 mM ionic strength, the buffers consisted of 100 mM sucrose, 1.3 mM KCl, 1 mM LM, and 10 mM CHES NaOH, pH 9.75, while at pH 9.75 and 250 mM ionic strength, the buffer was 100 mM sucrose, 210.9 mM KCl, 1 mM LM, and 45 mM CHES NaOH, pH 9.75.

For each individual assay, the initial absorbance level of ferrocytochrome *c* was determined at 550 nm, and the assay was initiated by the addition of LM-solubilized oxidase to a final concentration of 40 nM. Ferrocytochrome *c* oxidation was monitored at 550 nm at 25 °C for 2 min on a Hewlett-Packard model 8453 diode array spectrophotometer. Ferrocytochrome *c* concentration was varied from 0.4 to 33 μM.

The pH dependence of cytochrome *c* oxidase and peroxidase activities was measured at 550 nm using oxidase reconstituted in phospholipid vesicles in a buffer containing 100 mM sucrose, 100 mM KCl, 25 μM ferrocytochrome *c* and either 10 mM (H)EPPS, NaOH, pH 8.5; 10 mM CHES, NaOH, pH 9.0; 10 mM CHES, NaOH, pH 9.5; or 10 mM CHES, NaOH, pH 10.0. 10 μM valinomycin and 5 μM CCCP were included in the medium to dissipate any gradients formed during the assay. 150 mM H_2O_2 was included as a substrate for the peroxidase assays and all peroxidase rates were corrected for non-enzymatic oxidation of ferrocytochrome *c* (usually 25% of the total rate). At pH values below 9.0, the non-enzymatic oxidation rates of cytochrome *c* were greater than the oxidase-catalyzed rate.

The steady-state kinetics of hydrogen peroxide interaction with COX in liposomes was monitored at 550 nm using 25 μM ferrocytochrome *c*, 100 mM KCl, 100 mM sucrose, 10 μM valinomycin, 5 μM CCCP and various concentrations of H_2O_2 (10–500 mM). All assays were corrected for non-enzymatic oxidation of ferrocytochrome *c* (25–38% of the rate). Hydrogen peroxide concentration was determined by using an extinction coefficient of 40 mM^{-1} cm^{-1} at 250 nm [15].

2.3. Steady-state heme a reduction levels

Heme reduction levels were monitored at 444−460 nm for hemes (a+a_3) and at 605−620 nm for heme *a* [32]. At pH 7.8, the buffer contained 100 mM sucrose, 1 mM LM, 7 mM ascorbate, 0.2 mM TMPD, 1.25 nM cytochrome *c*, and 10 mM HEPES, NaOH, pH 7.8, while at pH 9.75, the buffer was comprised of 100 mM sucrose, 1 mM LM, 7 mM ascorbate, 0.2 mM TMPD, 2 nM cytochrome *c*, and 10 mM CHES, NaOH, pH 9.75.

For individual assays, the substrates and buffer were added to the cuvette and then LM-solubilized oxidase was added to a concentration of 0.6 μM. Absorbance at 444−460 nm was followed spectrophotometrically for 5 min. The reduction level of the hemes is expressed as a percentage of the ratio of the 444−460- or 605−620-nm absorbance under aerobic steady-state conditions to the fully (dithionite) reduced absorbance at this wavelength. The turnover number of the oxidase is defined as moles of ferrocytochrome *c* oxidized per mole of oxidase per second.

2.4. Circular dichroism spectroscopy

COX was first incubated in LM [5 mg LM/mg protein] and then diluted to 5 μM COX in one of the following buffers: either 25 mM HEPES, NaOH, pH 7.0, or 7.4; 25 mM (H)EPPS, NaOH, pH 8.0; 25 mM CHES, NaOH, pH 9.0, or 9.75; or 25 mM CAPS, NaOH, pH 10.0 or 11.0, supplemented with 1 mM LM and either 90 or 95 (at pH 11) mM KCl.

Measurements were performed in quartz CD cuvettes with path lengths of 1 mm (for protein) and 10 mm (for heme Soret). All CD spectra were recorded at 18 °C as single scans (10 nm/min) using a Jasco J-500A spectropolarimeter.

3. Results

3.1. The effect of pH on the peroxidase and oxidase activities

Using the assay conditions described above, as the pH of the medium was raised from 8.5 to 10.0, with substrate concentration and ionic strength held constant, cytochrome *c* oxidase activity (which is 650 s^{-1} at pH 7.0) decreased by one order of magnitude, from 37 to 3 s^{-1}, while the peroxidase activity remained relatively constant (30–40 s^{-1}) (Fig. 2). The dependence of the reaction rate on the concentration of ferrocytochrome *c* was monitored spectro-

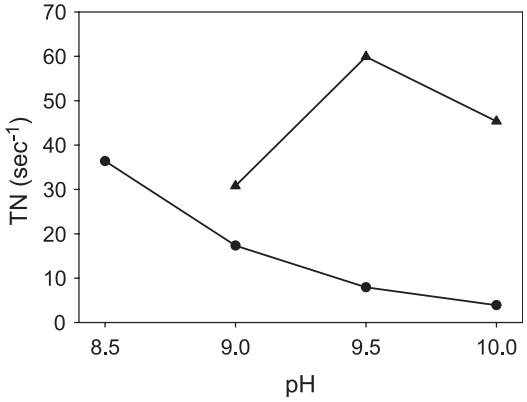

Fig. 2. The pH dependence of COX-catalyzed oxidase and peroxidase activities. Ferrocytochrome *c* (29 μM) oxidation by COX in phospholipid vesicles (2.5 nM) at different pH values was assayed using either oxygen (circles) or hydrogen peroxide (150 mM, triangles) as substrates.

photometrically at 550 nm at pH 7.8, at which extensive studies have been performed previously [33], and at pH 9.75.

At pH 7.8 under low ionic strength conditions (10 mM), the rate of ferrocytochrome *c* oxidation by bovine oxidase was biphasic on Eadie–Hofstee plots (Fig. 3A), as shown previously by Ferguson-Miller et al. [33]. The apparent high affinity site had an apparent K_m value of 0.66 μM and a V_{max} value of 100 s^{-1}, while the combined low and high affinity sites had an apparent K_m value of 7.2 μM, with a V_{max} value of 194 s^{-1} in qualitative agreement with Ferguson-Miller et al. [33] and Sinjorgo et al. [21].

At pH 9.75, low ionic strength conditions produced a qualitatively similar biphasic Eadie–Hofstee plot as that obtained at pH 7.8 (Fig. 4A). The high affinity site yielded an apparent K_m of 1.0 μM and a V_{max} value of 5.9 s^{-1}, while the combined high and low affinity sites had an apparent K_m value of 10.7 μM and a V_{max} value of 15 s^{-1}. The similarity of the biphasic Eadie–Hosfstee plots and the K_m values observed at pH 7.8 and pH 9.75 under low ionic strength conditions suggests that at low ionic strength, the ability of cytochrome *c* to interact with bovine COX is unaffected by pH, but the rate of electron transfer is reduced.

At pH 7.8 under high ionic strength conditions (250 mM), the Eadie–Hofstee plot was monophasic (Fig. 3B),

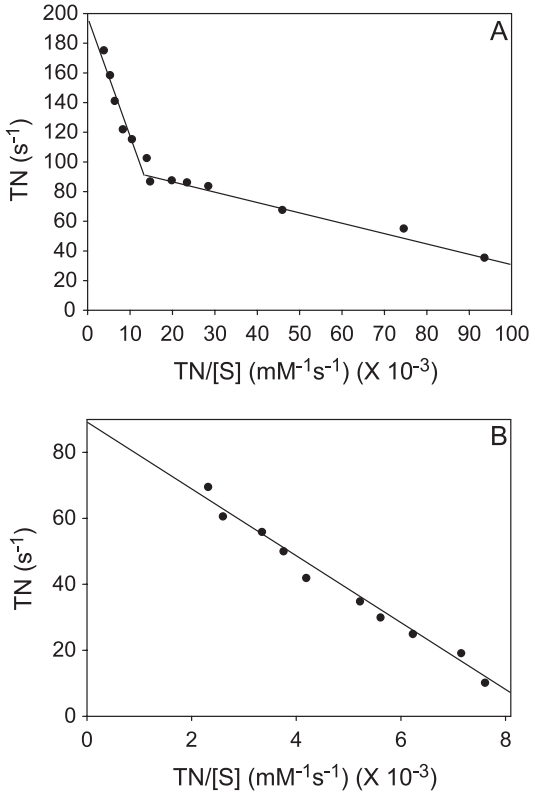

Fig. 3. Eadie–Hofstee plots of steady-state cytochrome *c* oxidation by bovine COX at pH 7.8 under low (A) and high (B) ionic strength conditions. Panel A indicates assays performed in a low ionic strength buffer (*I*=0.01) and Panel B indicates assays performed in a high ionic strength buffer (*I*=0.25) at pH 7.8. Each plot represents the results of three separate experiments. For each data point, three assays were performed per experiment. Values for K_m and V_{max} were estimated by linear regression.

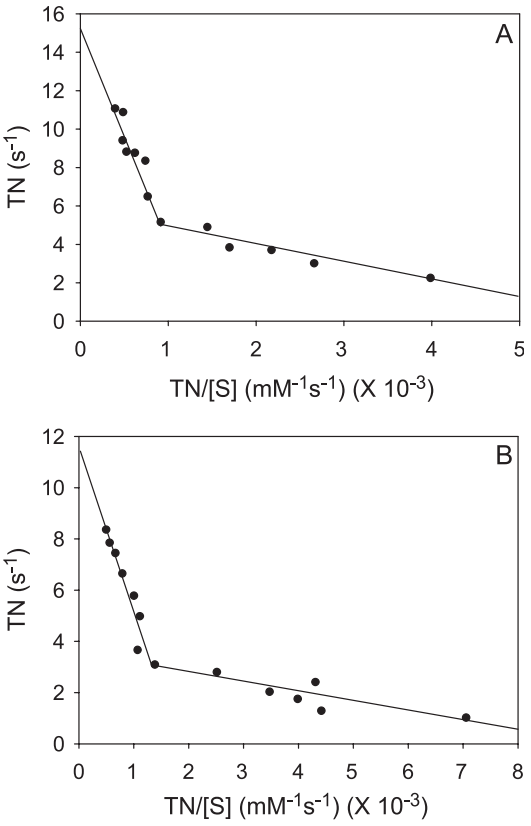

Fig. 4. Eadie–Hofstee plots of steady-state cytochrome *c* oxidation by bovine COX at pH 9.75 under low (A) and high (B) ionic strength conditions. Panel A indicates assays performed in a low ionic strength buffer (*I*=0.01) and Panel B indicates assays performed in a high ionic strength buffer (*I*=0.25) at pH 9.75. All other conditions were as described in Fig. 2.

also in agreement with the findings of Ferguson-Miller et al. [27]. The K_m value was 10 μM, and the V_{max} was 89 s^{-1}. These results are comparable to those reported by Sinjorgo et al. [21] who obtained K_m of 22 μM and V_{max} of 210 s^{-1}.

High ionic strength conditions at pH 9.75 (250 mM) yielded a biphasic Eadie–Hofstee plot (Fig. 4B), with values for apparent K_m and V_{max} qualitatively similar to those observed under low ionic strength conditions at pH 9.75. The high affinity site had an apparent K_m value of 0.38 μM, with a V_{max} value of 4 s^{-1}, while the combined high and low affinity sites had an apparent K_m value of 6.1 μM and a V_{max} value of 11 s^{-1}.

Previous work [31] has shown that equine heart cytochrome *c* exhibits a pH-dependent conformational transition near pH 9.0 and that this alkaline conformational form of cytochrome *c* only exists in the oxidized form. The kinetics of the formation of the alkaline form of ferricytochrome *c* was monitored during our assays, and it was determined that neither the extent nor the rate of formation of the alkaline form correlated with oxidase electron transfer.

These results indicate that the low cytochrome *c* oxidase activity at pH 9.75 is not due to an effect on the conformation of cytochrome *c* or due to a change in the

strength of the interaction between cytochrome *c* and the oxidase.

3.2. Steady-state kinetics of cytochrome c peroxidase activity using hydrogen peroxide as a substrate

The steady-state kinetics of the peroxidase activity was examined as a function of the concentration of H_2O_2 at pH 9.75, monitoring the oxidation of ferrocytochrome *c*. The oxidase was incorporated into phospholipid vesicles to stabilize the subunit composition of the enzyme at alkaline pH values, as this pH has been shown to dissociate subunits (III, VIa, and VIb) from the solubilized enzyme [26]. In addition, hydrogen peroxide has been shown to cause chemical modification of the enzyme during long term incubations [34]. The oxidase activity of the enzyme reconstituted in phospholipid vesicles exhibited a respiratory control ratio of 3–5, even at pH 9.75, showing that at pH 9.75, the oxidase can establish a transmembrane electrochemical potential. At pH 9.75, the cytochrome *c* peroxidase activity was at least 10-fold higher than the oxidase activity (Fig. 2).

Fig. 5 shows that at pH 9.75, the peroxidase activity yields a biphasic Eadie–Hofstee plot upon varying the concentration of H_2O_2. This analysis results in apparent K_m values of 40 and 300 mM, and corresponding V_{max} values of 30 and 110 s^{-1}. The origin of the biphasic behavior is unknown.

3.3. Steady-state reduction levels of hemes a and a_3 at pH 7.8 and pH 9.75

It is well known that the rate of electron transfer from heme *a* to the heme a_3/Cu_B binuclear center is pH-dependent [35] and decreases at alkaline pH. In order to investigate the possibility that this process might be rate-limiting under the assay conditions used in the current work,

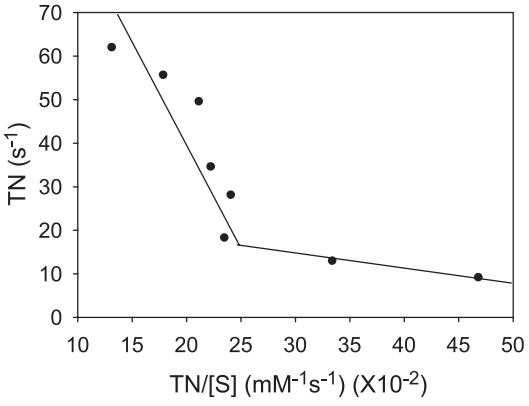

Fig. 5. Eadie–Hofstee plot of steady-state cytochrome *c* oxidation by bovine COX at pH 9.75 using hydrogen peroxide as a substrate. Ferrocytochrome *c* (27 μM) oxidation by COX in phospholipid vesicles (2.5 nM) at pH 9.75 was assayed using various concentrations of hydrogen peroxide (20–500 mM) as the substrate.

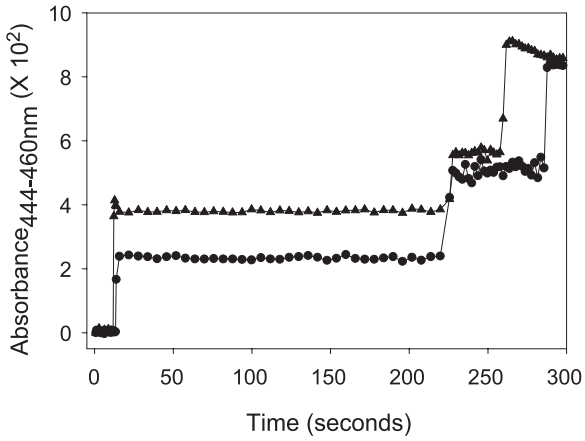

Fig. 6. Time course of the steady-state reduction levels of heme aa_3 of COX at pH 7.8 and pH 9.75. Heme reduction levels were monitored continuously at 444−460 nm [32], using oxidized COX as a spectrophotometric blank. The initial absorbance increase (at ~10 s) reflects the addition of COX, the second absorbance increase (at ~220 s) reflects the onset of the anaerobic state, and the final increase reflects the addition of dithionite in order to completely reduce the enzyme. The circles indicate COX assayed at pH 7.8, while the triangles indicate COX assayed at pH 9.75.

the steady-state reduction level of hemes *a* and a_3 was monitored by the absorbance at 444−460 nm at both pH 7.8 and 9.75 with COX undergoing a similar turnover number (8–10 s^{-1}) at both pH values [36]. Fig. 6 shows that at both pH 7.8 and 9.75, the aerobic steady-state lasted approximately 210 s, at which point all dioxygen in the cuvette had been consumed and the reaction became anaerobic. At pH 7.8, the aerobic steady-state level of reduction of the heme $a+a_3$ was approximately 25%, whereas at pH 9.75, the reduction level of the hemes was 40% of the fully reduced enzyme. Similar results (approximately 30% and 60% reduction levels at pH 7.8 and 9.75, respectively) were obtained when the absorbance at 605 minus 620 nm (to correct for TMPD absorbance [48]) was monitored; the absorbance at 605 nm is a qualitative measure of heme *a* reduction levels (data not shown). The data shown in Fig. 6 most likely underestimate the steady-state reduction level of

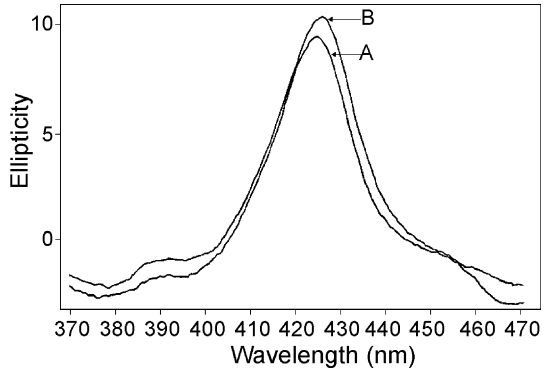

Fig. 7. Visible circular dichroism spectra of COX at pH 7.8 and pH 9.75. In trace A, the CD spectrum of COX (5 μM) in the Soret region was performed at pH 7.8, while in trace B, the CD spectrum was performed at pH 9.75. The units for ellipticity are mDeg.

heme *a* due to both heme *a* and a_3 contributing equally to the absorbance of dithionite reduced COX at 444 nm [28]. These results suggest that in the steady-state, the internal rate of electron transfer from heme *a* to heme a_3 is lower at alkaline pH and is rate-limiting for the oxidase reaction.

3.4. Circular dichroism spectroscopy

The increase in heme *a* reduction level at alkaline pH (Fig. 6) and the decrease in electron transfer rate (Fig. 2) suggest the possibility that alkaline pH induces a conformational change and possibly partially denatures the oxidase by changing protein–protein interactions or by removing subunits. This possibility was investigated by using circular dichroism spectroscopy in the UV region to detect conformational changes in the protein backbone, and also in the Soret (visible) region to detect changes in the heme environment.

The CD spectra in the far UV region of oxidized bovine COX solubilized in LM at pH 7.4 and pH 9.75 did not show any significant pH dependence (data not shown), indicating no large change in secondary structure at this pH. Also, SDS-PAGE showed that there was no loss of subunits from the enzyme at pH 9.75 (data not shown), emphasizing that no large change in the enzyme′s tertiary structure occurred.

CD spectra of the oxidized enzyme in the Soret region are shown in Fig. 7. At pH 7.4, the CD spectrum of the oxidized enzyme has a strong positive transition at 426 nm, in agreement with the results obtained by Hill et al. [37]. After a 1-h incubation in LM at 9.75, the peak was red-shifted by 1.5 nm to 427.5 nm. Similar results were reported by Orii et al. [38]. Most important is that when the enzyme was returned to pH 7.4, the CD band returned to its position at 426 nm (data not shown). Hence, for incubation times up to 1 h, at least, the effect of pH on the heme environment is fully reversible, as is the effect of pH on the oxidase activity. The pH dependence of the red shift in the CD spectrum is shown in Fig. 8.

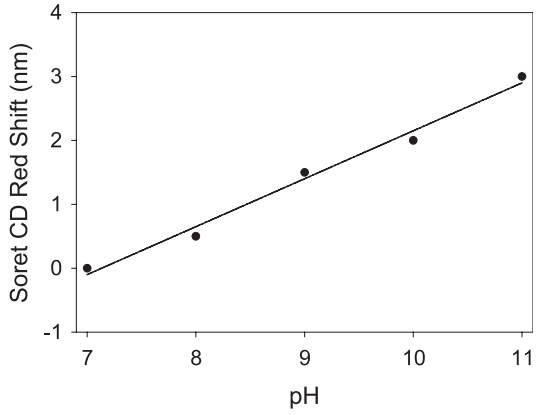

Fig. 8. The effect of pH on the position of the Soret maximum of the CD spectrum of COX. CD spectra were performed in buffers at different pH values as described in Section 2.

4. Discussion

The results presented show that the low oxidase activity of the enzyme at pH 9.75 is not due to any of the following: (1) the alkaline conformation of cytochrome *c*; (2) pH-induced denaturation of the oxidase; (3) decreased interaction strength between cytochrome *c* and the oxidase.

When assayed under low ionic strength conditions (10 mM) at a similar turnover number (8.0 s^{-1} to 10.0 s^{-1}), we found that the steady-state reduction level of heme *a* increased about 1.6- to 2-fold at pH 9.75 (~60%) as compared to pH 7.8 (~30%). Previous work with bovine heart COX solubilized in LM [39,40] or Tween-80 [41] has shown that electron transfer from heme *a* to heme a_3 is the internal rate-limiting step in COX turnover, and that electron transfer rates in both the bovine enzyme and the homologous bacterial (*R. sphaeroides*) oxidase are controlled by proton uptake in a tightly linked manner [42,43]. Hence, it is likely that the decreased electron transfer rate at pH 9.75 from heme *a* to heme a_3 in the current work is caused by a rate-limiting, redox-linked proton uptake by the enzyme.

The fact that the peroxidase activity of the enzyme is not reduced at alkaline pH strongly suggests that the limiting proton uptake is via the K-channel. Previous studies [15,16] have clearly demonstrated that the K-channel is not required for the reaction of the oxidase with H_2O_2, shown schematically in Fig. 1. In contrast, decreased proton uptake via the D-channel would be expected to inhibit both the oxidase and peroxidase activities of the enzyme.

Previous reports have demonstrated pH-dependent change in the heme optical properties [44,45]. It is shown in the current work that the alkaline pH-induced 1.5-nm red shift in the Soret CD transition is reversible. This could be due to a change in the protonation state of an amino acid residue or residues (or the propionate groups of the heme itself) in the vicinity of one or both of the hemes. It is not known whether the changes in the heme environment are related to the observed slow rate of proton delivery through the K-channel to the active site.

It is important to note that our Yonetani preparation of the bovine oxidase [23] yields a monomeric enzyme in detergent solution [24]. Recent work has demonstrated that bovine oxidase which has been “monomerized” by either alkaline treatment or with high concentrations of Triton X-100 has distinct kinetic properties in single-turnover experiments [25]. However, monomeric enzyme retains high steady-state electron transfer activity [46,47] and exhibits normal proton-pumping activity [26,32] when reconstituted into phospholipid vesicles.

5. Conclusion

It is concluded that at pH 9.75, the activity of the “monomeric” bovine oxidase is severely inhibited primarily due to a limited ability to provide protons through the K-

channel. Proton flux through the D-channel may also be impaired, but under the conditions studied in this work, it is the proton transit through the K-channel that is rate-limiting. As a result, the rate of reduction of the fully oxidized binuclear center ($O \rightarrow R_2$ in Fig. 1) is inhibited. The characteristics of the bovine oxidase at this high pH, therefore, mimic the behavior of the bacterial oxidase with mutants that block the K-channel.

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